THE USE OF AFFINITY CHROMATOGRAPHY FOR THE PURIFICATION OF AFFINITY LABELED PEPTIDES FROM STAPHYLOCOCCAL NUCLEASE

Meir WILCHEK
Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

We have recently described the affinity labeling of staphylococcal nuclease with bromoacetylated derivatives of competitive inhibitors [1]. The identification of the uniquely labeled residues was done after purification by the conventional techniques of peptide fractionation, requiring many steps of chromatography and electrophoresis.

In the following we describe the isolation of these peptides in essentially one step, by using our recently developed method for the isolation of labeled peptides from affinity labeled protein [2]. Staphylococcal nuclease attached to Sepharose is used as the stationary phase for affinity chromatography [3]. The affinity labeled peptide is the only one in a proteolytic digest which recognizes the enzyme and is bound, the other peptides passing unretarded through the column. The use of this method is illustrated by the isolation of affinity labeled peptides from staphyloccocal nuclease reacted with bromoacetyl and diazonium derivatives of deoxythymidine-3′-*p*-aminophenyl-phosphate-5′-phosphate, and with bromoacetyl-*p*-aminophenyl-phosphate.

2. Material and methods

Staphylococcal nuclease was a gift from Dr. C.B. Anfinsen. The deoxythymidine 3′,5′-diphosphate (pdTp) was purchased from Calbiochem, and deoxythymidine 3′-*p*-aminophenyl-phosphate 5′-phosphate, (pdTp-aminophenyl) deoxythymidine 3′-^{14}C-bromoacetyl-*p*-aminophenyl-phosphate (Reagent I) and ^{14}C-bromoacetyl-*p*-aminophenyl-phosphate (Reagent II) were prepared as described previously [4]. The deoxythymidine 3′-*p*-diazophenyl-phosphate 5′-phosphate (Reagent III) was prepared from pdTp-aminophenyl by treatment with nitrous acid and used immediately in the modification reaction.

The Sepharose-nuclease column [5] was prepared by coupling nuclease to Sepharose 4B which had been activated with cyanogen bromide [6]. Amino acid analyses were performed according to Spackman et al. [7]. Samples were hydrolyzed in constant boiling HCl in evacuated, sealed tubes at 110° for 20 hr.

The reaction mixtures used for affinity labeling experiments contained low concentration of nuclease (10^{-5} M). Protein concentration was determined spectrophotometrically at 280 mμ using $E^{1\%}_{1\,cm}$ of 9.30 [8]. The concentration of the bound azo nucleotide was determined spectrophotometrically at 342 mμ.

3. Results

Staphylococcal nuclease was affinity labeled with reagent I as described previously [1]. The alkylated protein was digested with trypsin (1% by weight) at 37° in 0.05 M borate buffer, pH 8. After 3 hr the solution was adjusted to 10 mM $CaCl_2$ and the digest applied to a nuclease-Sepharose column containing 5 mg (0.3 μmole) of bound enzyme. The inhibitor capacity of which was determined with pdTp to be 0.1 μmole 30% of the theoretical value.

Fig. 1 demonstrates that no peptide labeled with reagent I emerged from the column when the

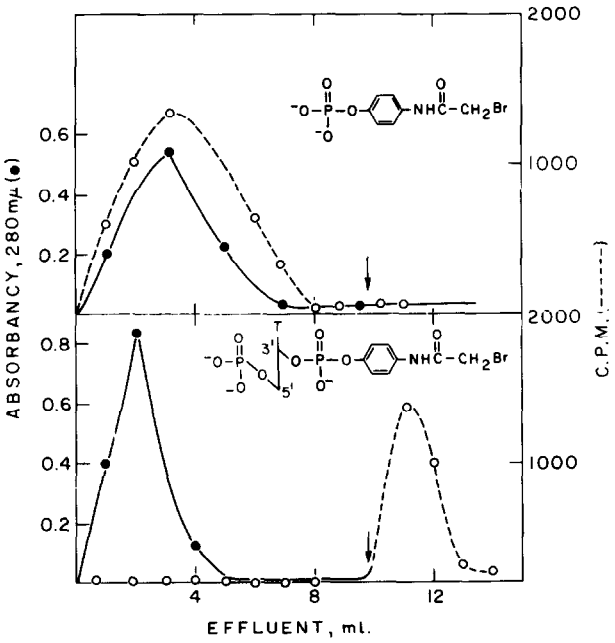

Fig. 1. Affinity chromatography on Nuclease-Sepharose column (0.5 × 2 cm) of affinity labeled peptides with reagents I and II. The columns were equilibrated with 0.05 M borate buffer, pH 8.0 containing 10 mM $CaCl_2$. Tryptic digest of modified nuclease (1.7 mg) were applied in 0.5 ml of the same buffer. After 10 ml of buffer had passed through, the bound peptides were eluted with NH_4OH, pH 11.0 (arrow).

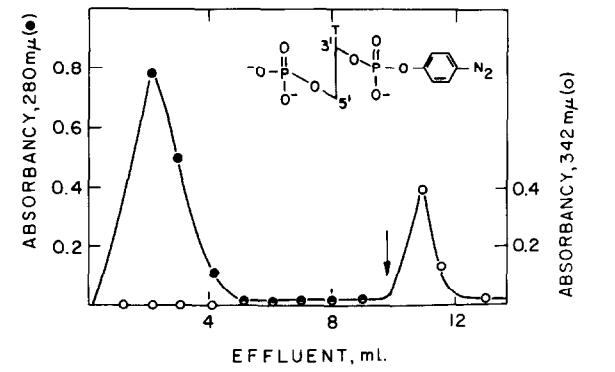

Fig. 2. Affinity chromatography on Nuclease-Sepharose column (0.5 × 2 cm) of affinity labeled staphylococcal nuclease with a threefold molar excess of reagent III. Tryptic digest of modified protein (1.7 mg) was applied to the equilibrated column (fig. 1). The column was developed with the same buffer. The bound peptide was eluted with NH_4OH, pH 11 (arrow).

column was washed with 0.05 M borate buffer, pH 8.0 containing 10 mM $CaCl_2$. The radioactive peptide which was bound to the column was eluted with NH_4OH pH 11. After elution, the column was washed immediately with 0.05 M borate buffer pH 8 and was used again. The yield of radioactive peptide was more than 80% of the amount applied to the column.

Amino acid analysis of the radioactive peptide showed that lysine 48 and lysine 49 were alkylated as was found previously on material isolated by several steps of chromatography, electrophoresis and radioautograms [1].

Satphylococcal nuclease reacts under similar conditions with reagent II which has a very poor affinity for the enzyme.

When the tryptic digest of nuclease modified with reagent II was applied on the Nuclease-Sepharose column, all peptides – including the radioactive one – emerged from the column in the early peak. No peptide could be eluted with NH_4OH, pH 11 (fig. 1), indicating that only ligands with high affinity for the bound enzyme can be used in this procedure.

The reaction of staphylococcal nuclease with threefold molar excess of reagent III at pH 8 in the presence of Ca^{2+} for 10 min was shown to be specific and stoichiometric by means of enzymatic inactivation and ultraviolet spectrum. The enzymatic inactivation could be prevented by addition of the strong competitive inhibitor pdTp, as well as by omitting Ca^{2+} which is required for binding of this inhibitor. The modified protein had a new absorption peak at 342 mμ, which was similar to *N*-acetyl tyrosine amide reacting with reagent III. A tryptic digest of the modified nuclease was run through the Nuclease-Sepharose column. None of the peptide labeled with reagent III emerged from the column (fig. 2). The yellow peptide which was bound to the column was eluted with NH_4OH, pH 11. After purification by one paper electrophoresis, pH 3.6, the yellow peptide was hydrolyzed. The amino acid analysis was consistent with residues 114–127 in the amino acid sequence of nuclease. Only tyrosine (115) in this sequence is susceptible to reaction with reagent III.

4. Discussion

We have recently described the selective purification of certain enzymes [3, 9], and other biological macromolecules by affinity chromatography [10]. The protein to be purified is passed through a column containing a cross-linked polymer or gel to which a specific competitive inhibitor or ligand of the protein has been covalently attached. In this study the reverse approach was taken and a protein-Sepharose column was used to purify specific ligand-bound peptides isolated from affinity labeled staphylococcal nuclease. The method is not limited to nuclease alone and has been successfully applied to the purification of peptides from goat anti dinitrophenyl-antibodies labeled by ^{14}C-1-bromoacetyl, 1'-DNP ethylenediamine [11], and for affinity labeled peptides derived from pancreatic ribonuclease A [12].

There are many advantages of using protein-Sepharose column for specific purification of their ligands; the operation is very fast, the peptides are concentrated from large volumes, and the column can be used several times. Only ligands having a high affinity for the protein will be absorbed on the protein-Sepharose column. Ligands having poor affinity will emerge together with the other peptides, as was shown above with bromoacetyl-*p*-aminophenylphosphate. It is possible that the unfavorable affinity constant of low affinity ligands can be compensated for by coupling a very large amount of enzyme to Sepharose. The procedures outlined and illustrated in this communication should be of value in the purification and isolation of small molecules which can reversibly and specifically bind to macromolecules, if the latter are not chemically altered during their binding to Sepharose or during the reversible absorption process.

References

[1] P.Cuatrecasas, M.Wilchek and C.B.Afinsen, J. Biol. Chem. 244 (1969) 4316.
[2] D.Givol, Y.Weinstein, M.Gorecki and M.Wilchek, Biochem. Biophys. Res. Commun., in press.
[3] P.Cuatrecasas, M.Wilchek and C.B.Afinsen, Proc. Natl. Acad. Sci. U.S. 61 (1968) 636.
[4] P.Cuatrecasas, M.Wilchek and C.B.Afinsen, Biochemistry 8 (1969) 2277.
[5] G.S.Omenn, D.A.Ontjes and C.B.Afinsen, Nature 225 (1970) 189.
[6] R.Axen, J.Porath and S.Ernback, Nature 214 (1967) 1302.
[7] D.H.Spackman, S.Moore and W.M.Stein, Anal. Chem. 30 (1958) 1190.
[8] S.Fuchs, P.Cuatrecasas and C.B.Afinsen, J. Biol. Chem. 242 (1967) 4768.
[9] M.Wilchek and M.Gorecki, European J. Biochem. 11 (1969) 491.
[10] P.Cuatrecasas and M.Wilchek, Biochem. Biophys. Res. Commun. 33 (1968) 235.
[11] Y.Weinstein, M.Wilchek and D.Givol, Biochem. Biophys. Res. Commun. 35 (1969) 694.